

The Role of Interleukin-4 in the Neuroprotective Benefits from Social Interaction

Undergraduate Honors Thesis

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ABSTRACT

Stroke is a leading cause of death in the US and results in over \$73.7 billion in health care costs every year. Strong social support has been shown to reduce mortality in cardiovascular disease and stroke, yet little is known about the physiological mechanism underlying these health benefits. Extensive tissue damage following stroke results in part from activated microglia, the immune cells of the brain. Oxytocin, a hormone released during social interaction, increases expression of Cluster of Differentiation 200 (CD200), a neuronal marker known to suppress microglial activation. We hypothesize that Interleukin-4 (IL-4), an anti-inflammatory cytokine produced by T-cells, is involved in the oxytocin-mediated mechanism through which social interaction conveys neuroprotection by upregulating expression of CD200 and complement receptor 1-related gene/protein-y (Crry). Using an animal model of social interaction, we show that oxytocin, IL-4, CD200, and Crry are increased in the brains of pair housed mice relative to isolated mice and that exogenous administration of IL-4 *in vitro* upregulates neuronal CD200, Crry, and oxytocin receptor gene expression. However, IL-4 does not alter oxytocin gene expression. An absence of IL-4 also eliminates the increase in CD200R found in paired mice compared to isolated mice. These findings further elucidate the physiological mechanism by which social interaction is beneficial to one's health, and may lead to new methods to reduce the risk for and improving recovery from multiple diseases such as stroke and cardiac arrest.

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CHAPTER 1

INTRODUCTION

Stroke is the fourth leading cause of death in the United States, and the second leading cause of death globally. It is also a leading cause of morbidity among adults in the US, costing an estimated \$73.7 billion in 2010. A stroke occurs when brain tissue is abnormally perfused and results in neurological problems. Strokes can be divided into two major categories: ischemic and hemorrhagic. Ischemic strokes, which comprise the majority (87%), involve an obstruction in the vessels in the brain which block blood flow to that particular area. Hemorrhagic strokes (13%) are usually caused by a weakening in the blood vessel wall or an aneurysm, resulting in the rupture of the vessel. The blood spills into the subarachnoid space or primary brain tissue and compresses the surrounding tissue. In both types of strokes, the lack of proper blood flow can cause serious neurological damage, including headaches, changes in sensory perception, memory loss, and loss of unilateral muscle motor function. The type of symptoms is related to the area of the brain where the stroke occurred, and the severity is related to the extent of the damage.

During an ischemic stroke, the lack of blood flow severely reduces nutrients and oxygen to area neurons, causing them to die. These dying neurons release glutamate and pro-inflammatory cytokines into the surrounding environment to recruit immune cells. Glutamate, which is used as a neurotransmitter, has been shown to be toxic to central nervous system (CNS) neurons in high extracellular concentrations (Rothman, 2004). Within the CNS, the resident immune cell is the microglia, which is related to cells of the mononuclear phagocytic lineage. These cells continually survey the CNS

and are the first to respond in the case of an injury or abnormal event. In the absence of injury, microglia express two phenotypes, an M1 pro-inflammatory phenotype or an M2 deactivated phenotype. The M1 phenotype microglia, which express major histocompatibility complex class II (MHC-II) antigens, can be stimulated by administration of the pro-inflammatory cytokines interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α) (Kreutzberg, 1996). The glutamate and ATP released from dying neurons can also activate the microglia (Loane et al., 2010). These activated microglia can act as professional phagocytes and release cytotoxic substances such as free oxygen radicals, nitric oxide, and proteases. Neuronal death is often connected to activated microglia. In culture, microglia that are exposed to dying neurons begin secreting pro-inflammatory cytokines such as TNF α and interleukin-12 (IL-12) as well as increasing expression of MHC-II (Pais et al., 2008). The media from these cultures also induced death in fresh neurons (Pais et al., 2008). The M2 phenotype microglia, which have lower cytotoxicity, are stimulated by tumor growth factor- β 1 (TGF- β 1) and interleukin-4 (IL-4) (Kretzberg, 1996). The deactivated or resting microglia provide a surveillance function and play a role in tissue repair and wound healing. M2 microglia are also associated with higher production of anti-inflammatory cytokines and growth promoting factors such as brain derived neurotrophic factor (BDNF).

Neurons express a variety of membrane proteins to interact with microglia and macrophages. Two important proteins are Cluster of Differentiation 200 (CD200) and complement receptor 1-related gene/protein- γ (Crry). CD200 is expressed in a variety of cell types; however, the receptor is only expressed in cells of the myeloid lineage. The interaction between CD200 and its receptor is thought to regulate microglial activation.

Mice deficient in CD200 had significantly more circulating activated microglia (Hoek et al., 2000). Following injury of the facial nerve, CD200 deficient mice had a faster localized microglial response, indicating that CD200 inhibits microglial activation (Hoek et al., 2000). Treatment with anti-CD200 antibody resulted in reduced macrophage and microglia infiltration into the spinal cord (Chitnis et al., 2007). Increased expression of CD200 in neuronal cultures also confers protection from neurotoxicity from activated microglia through inflammation-induced damage. The protection was eliminated when anti-CD200 antibody was added to the culture (Chitnis et al., 2007). Under neuroinflammatory conditions, lower CD200 expression was correlated with higher microglial activation (Lyons et al., 2007).

Crry is a murine membrane bound complement regulator protein found in microglia, astrocytes, and neurons. It is a functional homologue of two complement inhibitors in humans, membrane cofactor protein and decay-accelerating factor. Expression is higher in microglia and only found at the RNA level in neurons (Davoust et al., 1999). Administering Crry protein and reducing complement activation leads to increase in neuron survival in a mouse model of traumatic brain injury (Leinhase et al., 2006). Crry expression is also associated with accumulation of degenerating neurons in a mouse model of Alzheimer's disease and fewer activated microglia (Wyss-Coray et al., 2002).

Within the time frame of an ischemic stroke, core neurons in the blood-deprived area are committed to die within hours as a result of decreased ATP production and glutamate toxicity (Kaushal et al., 2008). However, neurons in the penumbra may persist for days or weeks before apoptosis making these an ideal target for treatment

and therapy. Following an ischemic event, microglia are recruited to the site and release pro-inflammatory cytokines. This inflammatory response can persist for days, potentially causing more damage to neurons (Kaushal et al., 2008). Current treatments for stroke involve attempting to breakdown the blood clot to restore proper blood flow. An intravenous administration of recombinant tissue plasminogen activator (rtPA) is the recommended treatment however only 31%-50% of patients when treated within a 3 hour time frame showed improved recovery at 3 months compared to 20%-38% of patients in a placebo group. Additionally, rtPA has a 6% risk of intracranial hemorrhage (Moheet et al., 2013). To avoid these issues, a potential avenue for treatment is by targeting neurons in the penumbra. By reducing the inflammatory response and microglia activation, tissue damage could be lessened.

Social interaction has been implicated in reduced risk for cardiovascular disease, cancer, accidents, and suicide (Kawachi et al., 1996). Higher levels of social support are also correlated with improved function and faster recovery following stroke, particularly in severely impaired patients (Glass et al., 1993; Tsouna-Hadjis et al., 2000). The benefits of social interaction have been thoroughly studied in the clinic, although the physiological mechanism behind the effect is still under investigation. Recent studies point to oxytocin as a mediator in the response. Oxytocin (OT) is a hormone released by the neurohypophysis that is most widely known for its induction of uterine contractions and milk letdown. However, oxytocin also regulates mammalian social interactions and facilitates pair bonding, suggesting a potential role in mediating the physical benefits of social support. Exogenous administration of oxytocin has been shown to reduce infarct volume in a mouse model of focal cerebral ischemia (Karelina et al., 2011). Current

hypotheses of the mechanism involve the anti-inflammatory effects of oxytocin. In a model of ischemia/reperfusion injury, rats treated with oxytocin had improved renal function and damage along with decreased levels of inflammatory cytokines, such as TNF α , and free oxygen radicals when compared to control rats, suggesting that the anti-inflammatory effects of oxytocin improved outcome (Tugtepe et al., 2007). Previous research in our lab has shown that oxytocin increases expression of CD200 and Crry in the cortex, providing another pathway for neuroprotection (unpublished data).

Interleukin-4 (IL-4) is an anti-inflammatory cytokine secreted by helper T-cells to induce phenotype switching from a pro-inflammatory Th1 state to an anti-inflammatory Th2 state. These Th2 T-cells also secrete IL-4 and can help inhibit production of pro-inflammatory cytokines. Without IL-4, phenotype switching from Th1 to Th2 is disrupted and levels of the Th2 cytokines IL-5, IL-9, and IL-10 are reduced (Kopf et al., 1993). IL-4 deficient mice have higher expression of IL1- β in the hippocampus and cortex when the immune system is stimulated with lipopolysaccharide compared to wild type mice (Lyons et al., 2009). Aside from its effect on T-cells, IL-4 also exerts regulatory functions on macrophages and microglia which express the IL-4 receptor but do not produce IL-4. *In vitro*, IL-4 can induce proliferation of microglia, an effect which is neutralized by the addition of anti-IL-4 antibody (Suzumura et al., 1994). In a dose-dependent fashion, IL-4 increases activation of microglia, but suppresses the IFN γ induction of MHC-II expression on the cell surface (Suzumura et al., 1994). The reduction in antigen presenting activity may lower peripheral damage from the active microglia. When the immune system was stimulated with lipopolysaccharide (LPS), IL-4 deficient mice had greater numbers of circulating activated microglia compared to wild type control mice

(Lyons et al., 2009). IL-4 activated microglia can also induce neurogenesis and angiogenesis in adult neural progenitor cells (Butovsky et al., 2006). In cell cocultures of neurons and microglia primed with IFN γ , treatment with IL-4 was found to decrease neuronal cell injury in a dose-dependent manner by decreasing microglial production of TNF α and nitric oxide (Chao et al., 1993). In addition to its actions on microglia, IL-4 modulates inducible nitric oxide synthase in other glial cells, such as astrocytes (Paintlia et al., 2006). IL-4 also acts on neurons. Exogenous IL-4 has been shown to increase neuronal expression of CD200 compared to IL-4 deficient mice which have lower expression of CD200 (Lyons et al., 2007; Lyons et al., 2009). Neurons from wild type mice were able to attenuate the LPS induced pro-inflammatory changes in culture compared to neurons enriched from IL-4 deficient mice (Lyons et al., 2009). In a model of focal cerebral ischemia, mice without a functional IL-4 gene had increased infarct volumes and more impaired neurological behavior (Xiong et al., 2011). The multiple pathways through which IL-4 acts indicates its important role in neurological function and immune response.

Decades of clinical studies demonstrate that social interaction and support can reduce mortality and morbidity, although not much is known about the physiological mechanism behind the protective effects. Oxytocin, which has long been known to mediate pair bonding in mammals, is a likely target. The effect of oxytocin on levels of anti-inflammatory cytokines in the CNS suggests a connection between oxytocin and IL-4 in neuroprotection. Because of the actions of IL-4 in reducing pro-inflammatory cytokines and decreasing activated microglia, we hypothesize that IL-4 may play a role in the neuroprotective benefits of social interaction. Further study of the effects of social

interaction on IL-4 and oxytocin will lead to more advanced methods and treatments to improve recovery for stroke or other cerebral ischemic events.

CHAPTER 2

MATERIALS AND METHODS

Mice

Male C57/BL6 mice (6-8 weeks old) were obtained from Charles River Laboratories or Jackson Laboratories. The mice were randomly assigned to an experimental group and housed either individually (isolated) or with an ovariectomized female (paired) for 7 days.

Focal Cerebral Ischemia Model

Male mice underwent middle cerebral artery occlusion (MCAO) surgery with one hour of occlusion followed by reperfusion; the surgery was performed by a trained surgeon as previously described (Karelina et al., 2011.). The left hemisphere was occluded. Following surgery, core body temperature was maintained with a heating pad, and a score of observable neurological impairment was recorded. During recovery and until tissue collection, mice were paired or singly housed according to experimental group. Twenty-four hours after surgery, whole brain tissue was collected.

Analysis of Gene Expression by Quantitative Real-Time PCR

In one series of experiments, whole brain tissue was collected, placed in RNeasy® (Qiagen) immediately, and stored at -80°C for 3 days. Brains were dissected to isolate the hypothalamus, hippocampus, and cortex. mRNA was isolated using the RNeasy® Mini Kit from Qiagen and stored at -80°C. cDNA was generated by incubating mRNA with 1:10 diluted Random Primers (Invitrogen) and 10 mM dNTP (Invitrogen) at

65°C for 5 minutes, then adding 5X First-Strand Buffer (Invitrogen), 0.1M DTT (Invitrogen), and RNaseOUT (Invitrogen), incubating at 37°C for 2 minutes. Finally, M-MLV (Invitrogen) is added and incubated at 25°C for 10 minutes, then 37°C for 50 minutes, then 70°C at 15 minutes. Incubations were done in an i-Cycler from Bio-Rad Laboratories, Inc. Gene expression was analyzed using quantitative Real-Time Polymerase Chain Reaction on an Applied Biosystems 7500 Fast Real-Time PCR System. Probes were bought from Applied Biosystems.

Microglia Isolation

Collected tissue was homogenized and centrifuged at 1400rpm for 5 minutes at 15°C. The pellet was suspended in 3mL of 70% Percoll (GE Healthcare Life Science) diluted with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Life Technologies). The suspension was layered with 3mL 50% Percoll, then 3mL 35% Percoll, and finally 2mL HBSS before being centrifuged at 2000g for 30 minutes at 15°C. The microglia layer, an interphase between the 70% and 50% Percoll layers, was aspirated and centrifuged with 10mL HBSS at 1400rpm for 5 minutes at 15°C. The pellet was then resuspended with 450µL HBSS to obtain enriched microglial cells.

CATH.a Cell Culture

IL-4 and OT incubation experiments were conducted using the CATH.a cell line (ATCC® CRL-11179™). Established from a brain tumor in a transgenic mouse, the CATH.a cell line is neuronal in nature and can express dopamine and norepinephrine. It is immortalized and tumorigenic. Cells were grown in RPMI 1640 medium with 2mM L-

glutamine, 10mM HEPES, and 1.0mM sodium pyruvate (ATCC®) supplemented with 1.5g/L sodium bicarbonate (Sigma-Aldrich), 4.5g/L glucose (Sigma-Aldrich), 8% horse serum, and 4% fetal bovine serum. Cells were incubated in 25cm² Corning® cell culture flasks (Sigma-Aldrich) at 37°C with 5% CO₂. Cells were subcultivated at a ratio of 1:4 every 3 days using 0.12% trypsin without EDTA to remove adherent cells. After subcultivation, cells were grown to 70% confluency before beginning experimental procedures.

In one series of experiments, IL-4 (Bachem) was added to cells at a concentration of 100ng/mL and incubated for 4 hours. RPMI 1640 medium was added for control samples. mRNA was then isolated from the cells using the RNeasy® Micro kit and protocol from Qiagen. cDNA was generated using the protocol listed above for tissue samples.

In another series of experiments, oxytocin (Bachem) was added to cells at a concentration of 100µM. A separate group of cells received 100µM oxytocin and 100µM oxytocin antagonist (Bachem), while control cells received RPMI 1640 medium. All groups were incubated for 24 hours. mRNA was isolated via the RNeasy® Micro kit and protocol from Qiagen. cDNA was generated using the protocol listed above for tissue samples.

Analysis of Stroke Volume

Infarct volume was determined by staining with 2,3,5-triphenyltetrazolium chloride (TTC), which differentiates between living and dead tissues. The white compound is reduced to the red compound 1,3,5-triphenylformazan by dehydrogenase

enzymes important in cellular metabolism in the mitochondria. As a result, the infarct area appears white and live tissue appears pink or red.

Once removed, the brain is chilled at -20°C for 5 minutes and then cut in 2mm thick coronal sections resulting in 5 slices. The sections are incubated in 1% TTC (Sigma-Aldrich) solution created from 0.9% saline (Baxter Healthcare Corporation) for 2 minutes at 37°C. The sections are then inverted and incubated for 2 minutes. After a final inversion, the sections are incubated for 4 minutes before the 1% TTC solution is replaced with 10% formalin. After 24 hours, the infarct volumes are measured using Inquiry® Quantitative Autoradiography (Version 3.08, Loats Associates, Inc.) software.

Calculations and Statistics

Averages and standard errors were calculated using Microsoft Excel and SPSS (IBM, SPSS Inc.) A one-tailed student's t-test was used to determine significance, defined here as $p < 0.05$.

CHAPTER 3

RESULTS

Social interaction increases expression of IL-4

To determine whether gene expression of IL-4 was affected by social interaction, adult male mice were paired with an ovariectomized female mouse or singly housed for 1 week. Gene expression for IL-4 was examined by Real-Time PCR in the hippocampus, hypothalamus, and cortex. The paired mice had significantly elevated levels of IL-4 expression in the hippocampus and hypothalamus compared to the isolated mice (Figures 1A-B). In the cortex, there is a trend towards a significant increase in IL-4 expression in paired mice compared to isolated mice (Figure 1C). The high variability is due to low overall expression of IL-4 in the brain. The results indicate that social interaction results in an increase in IL-4 gene expression in multiple areas of the brain.

To determine whether an ischemic event results in the same pairing effect on IL-4 expression, adult male mice were paired with an ovariectomized female mouse or singly housed for 1 week before we performed MCAO surgery. The right hemisphere (contralateral hemisphere) was collected after 24 hours and gene expression was analyzed by qRT-PCR. The paired mice had increased expression of IL-4 compared to the isolated mice (Figure 1D) showing that the pairing effect lasts even after an ischemic event.

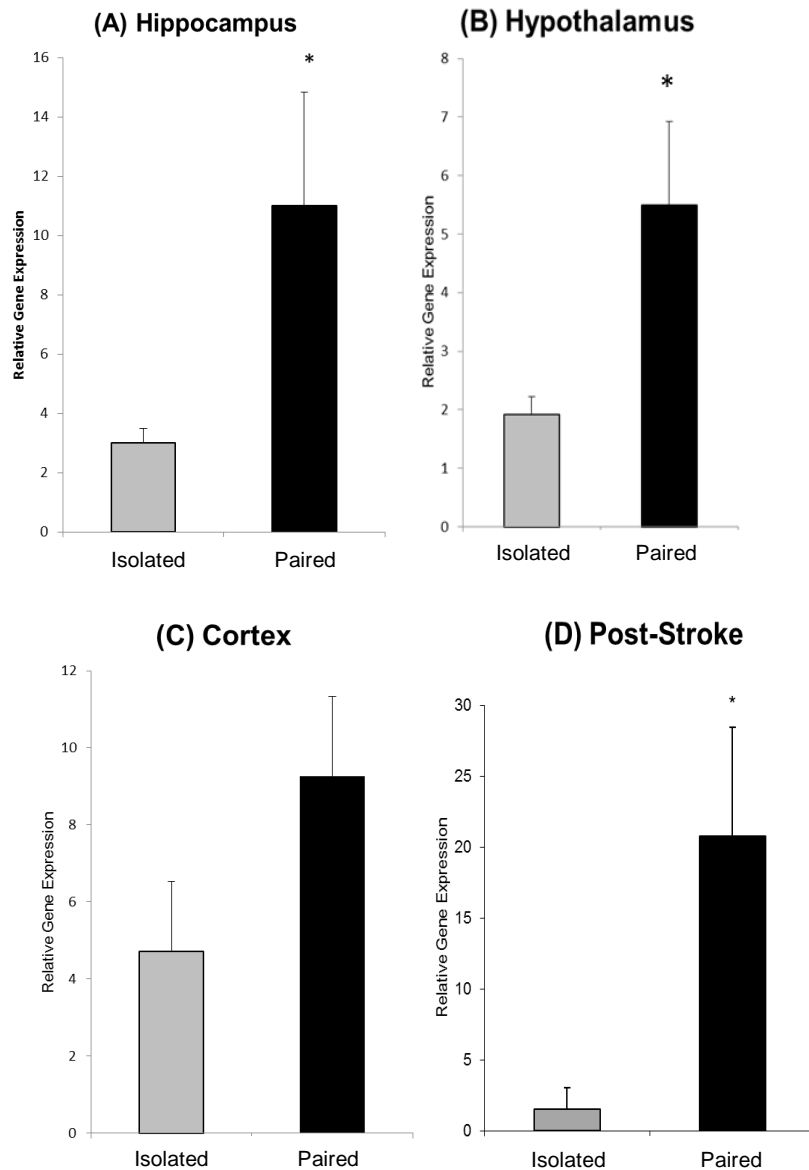


Figure 1A-C. Social interaction increases expression of IL-4. Male mice housed with a female mouse for 1 week had significantly increased levels of IL-4 gene expression in the hippocampus (A) and in the hypothalamus (B) (* $p < 0.05$; student's t-test; $n=10$). In the cortex (C), the difference in levels of IL-4 mRNA trends towards a higher expression in paired animals ($p < 0.06$; student's t-test; $n=10$). (D) Following MCAO surgery, paired mice had higher expression of IL-4 in the contralateral (non-stroke) hemisphere compared to isolated mice ($p < 0.05$; student's t-test; $n=7-8$).

IL-4 increases expression of protective markers on neurons.

To investigate the effect of IL-4 in the brain, we incubated CATH.a neuronal cells with IL-4 (100ng/mL) for 4 hours and looked at gene expression of CD200 and Crry. CD200 and Crry are glycoproteins which reduce activation of microglia which can increase infarct volume during an ischemic event. Cells treated with IL-4 had significantly increased expression of CD200 (Figure 2A) and Crry (Figure 2B) compared to the control group indicating a protective effect of IL-4 on neurons. This suggests that one potential pathway of the benefits of social interaction is through increased expression of neuronal protective markers by an increased level of IL-4.

IL-4 decreases expression of IL-4Ra but does not change OT expression

Because oxytocin has been shown to increase CD200 and Crry expression on neurons as well, we were interested in the effect of IL-4 on oxytocin expression. CATH.a neurons were incubated with exogenous IL-4 (100ng/mL) for 4 hours and gene expression was analyzed by qRT-PCR. IL-4 administration did not change the expression of oxytocin to an appreciable degree (Figure 3B). However, IL-4 could regulate oxytocin expression by way of its receptor. Expression of oxytocin receptor (OTR) was shown to be increased although the difference only trends towards significance (Figure 3A). The data suggests that IL-4 may regulate oxytocin signaling through expression of its receptor. IL-4 also downregulates the IL-4 receptor α (IL-4R α) (Figure 3C) indicating that negative regulation of the receptor is very tightly controlled.

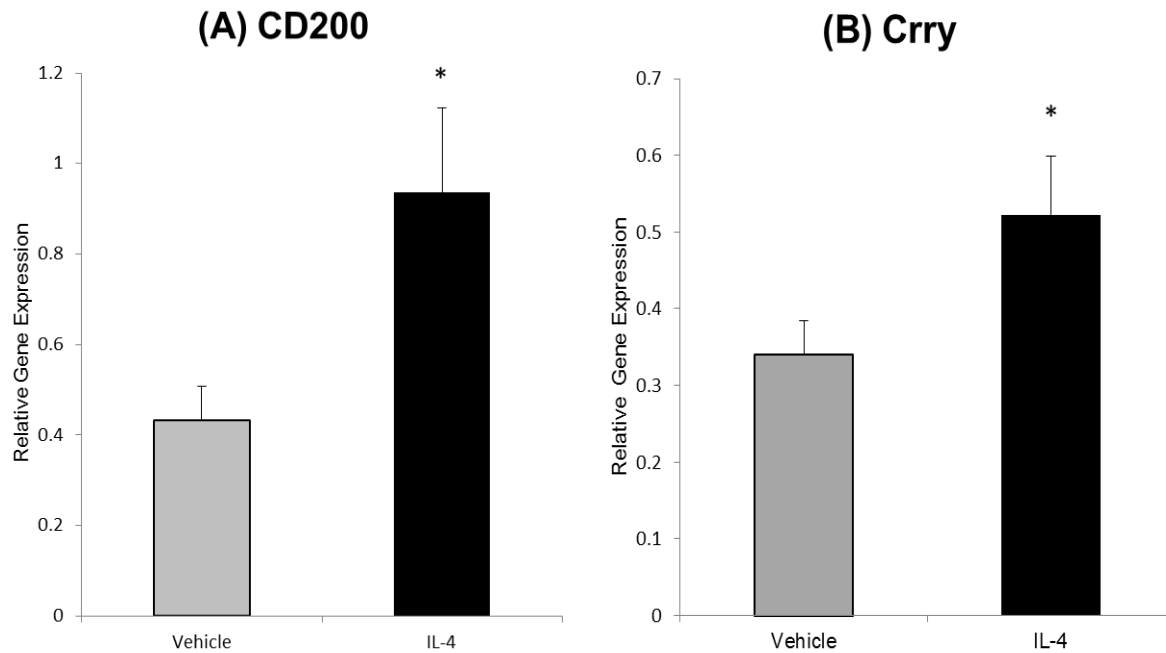


Figure 2A-B. Exogenous IL-4 increases expression of CD200 and Crry in neurons.

Neurons from the CATH.a cell line were incubated with exogenous IL-4 for 4 hours.

Cells showed significant increases in expression of CD200 (A) and Crry (B) following IL-4 incubation (* $p < 0.05$; student's t-test; $n=6$).

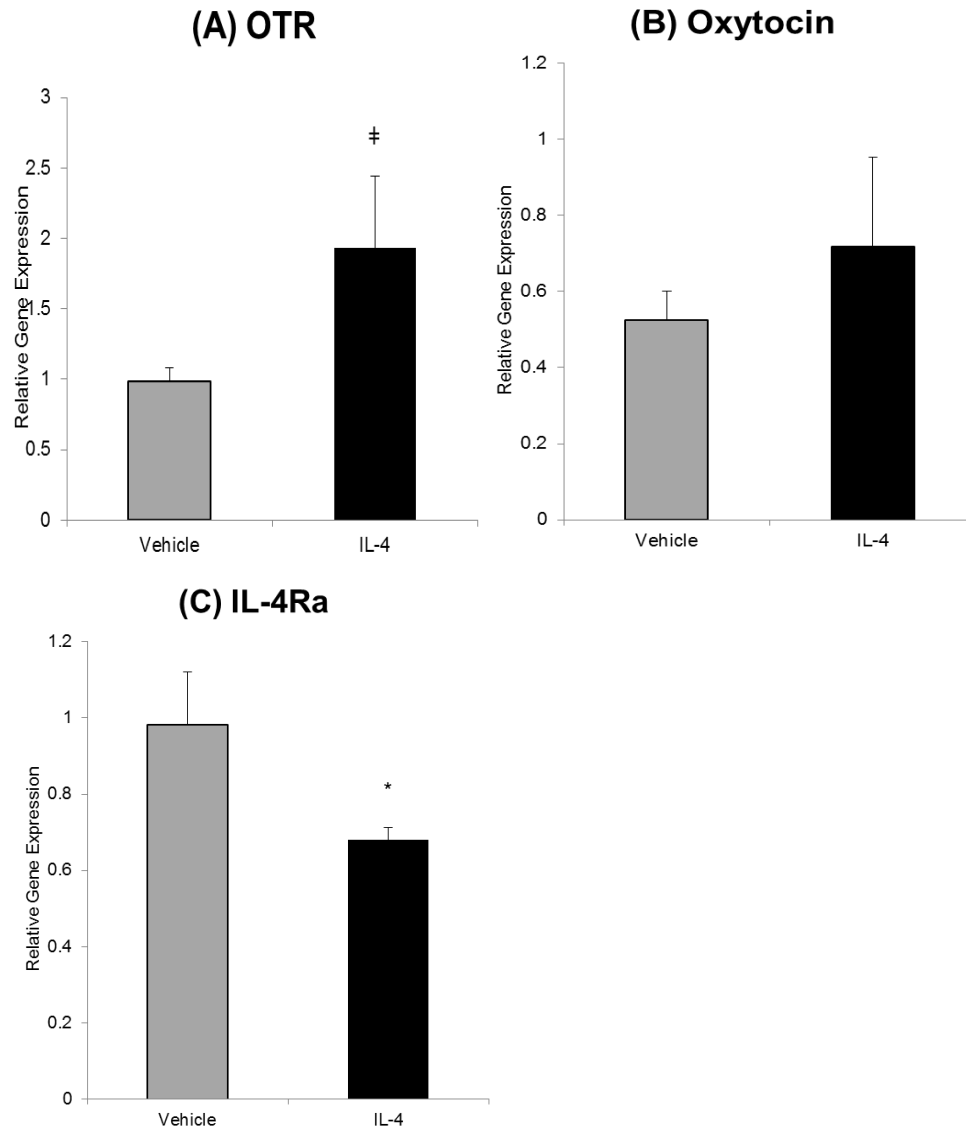


Figure 3A-C. Exogenous IL-4 affects expression of oxytocin receptor and IL-4 receptor but does not affect expression of oxytocin. Neurons from the CATH.a cell line were incubated with exogenous IL-4 for 4 hours and gene expression was analyzed by qRT-PCR. (A) IL-4 increases expression of OTR at a level that approaches significance ($p = 0.06$; student's t-test; $n=6$). (B) However, IL-4 had no significant effect on oxytocin expression in the neurons ($p = 0.23$; student's t-test; $n=6$). (C) IL-4 decreased expression of IL-4Ra compared to controls ($p < 0.05$; student's t-test; $n=6$).

Oxytocin changes IL-4R α but not IL-4 expression in neurons

Oxytocin is known to be increased following social interaction and plays a role in neuroprotection (Stuller et al., 2012). To determine the effect of exogenous oxytocin on neurons, CATH.a neurons were incubated with oxytocin (100 μ M) or oxytocin (100 μ M) with oxytocin antagonist (OTA) (100 μ M) for 24 hours. Gene expression was analyzed by qRT-PCR. Oxytocin administration had no effect on IL-4 expression in CATH.a neurons (Figure 4A). However, oxytocin increased expression of IL-4R α (Figure 4B) suggesting that regulation of IL-4 signaling is mediated through the receptor. Cells incubated with oxytocin and OTA also showed increased expression of IL-4R α (Figure 4B). OTA is a competitive inhibitor of OTR. The data provides evidence that with neurons OT may also bind to the vasopressin receptor instead of the oxytocin receptor to initiate change in gene expression; which would explain why the OTA was ineffective in preventing the effects of OT on IL-4R α .

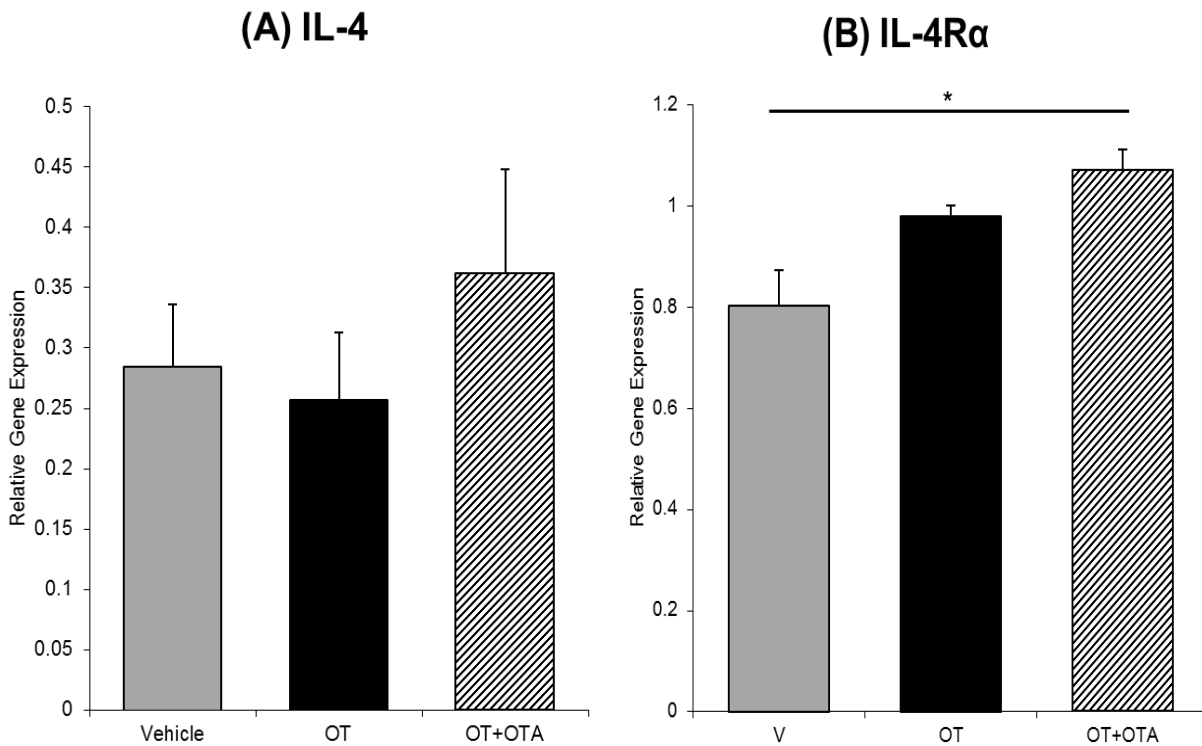


Figure 4A-B. Oxytocin changes expression of IL-4Rα but does not affect IL-4. CATH.a neurons were incubated with OT for 24 hours and gene expression was analyzed using qRT-PCR. (A) Oxytocin administration did not change expression of IL-4 compared to control groups ($p > 0.05$; ANOVA; $n=10$). (B) Oxytocin increased expression of IL-4Rα in CATH.a cells compared to the vehicle control group. When both oxytocin and oxytocin antagonist were administered, IL-4Rα expression increased compared to both the control and oxytocin only groups ($*p < 0.05$; ANOVA; $n=10$).

Social interaction decreases IL-4R α expression on microglia after stroke

Next we investigated the effect of social interaction on microglia. Adult male mice were housed either paired with an ovariectomized female mouse or singly housed for one week. Brain tissue was extracted, and microglia were isolated using a Percoll gradient centrifugation protocol (Henry et al., 2009). Gene expression was analyzed using qRT-PCR. Paired and isolated mice expressed similar levels of IL-4R α (Figure 5A). To assess whether an ischemic event would change expression levels, adult male mice were either paired or singly housed for one week before undergoing MCAO surgery. The left hemisphere was occluded for 60 minutes before reperfusion. Gene expression was determined in the same fashion as the previous experiment. Paired and isolated mice had similar levels of IL-4R α expression in the ipsilateral (stroke) hemisphere (Figure 5B); however, isolated mice had higher expression in the contralateral hemisphere (Figure 5B) which reflects a potential decrease in neural IL-4. Isolated animals also had significantly higher expression of IL-4R α in the contralateral hemisphere when compared to the ipsilateral hemisphere, a difference not reflected in paired animals (Figure 5B). The elevation suggests that the low expression of IL-4 in isolated mice causes an upregulation of its receptor following an ischemic event.

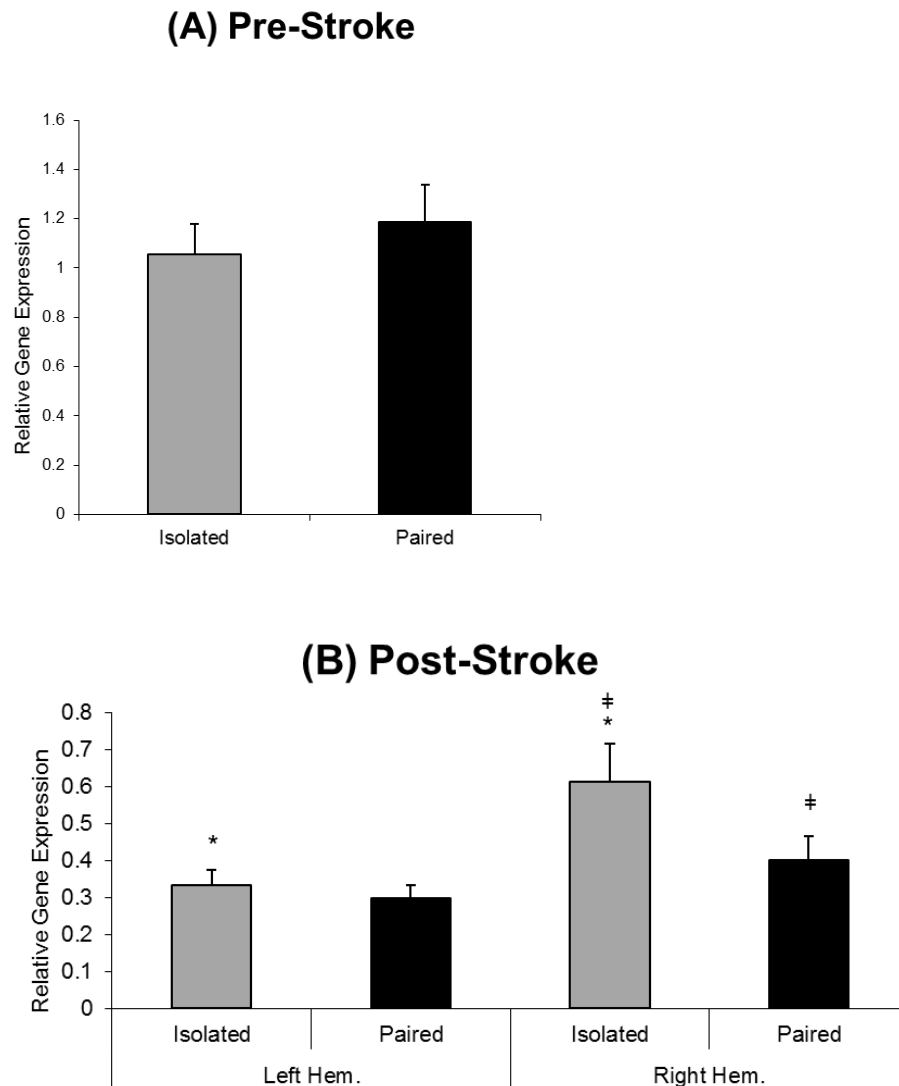


Figure 5A-B. Social interaction regulates IL-4R α expression on microglia following an ischemic event. (A) Prior to MCAO, there is no significant difference in IL-4R α expression on enriched microglia ($p=0.26$; student's t-test; $n=12$). (B) 24 hours after MCAO, isolated animals have higher expression of IL-4R α on the contralateral hemisphere ($^{\dagger}p < 0.05$; ANOVA; $n=10$). Isolated animals also had significantly increased expression of IL-4R α on the contralateral hemisphere compared to the ipsilateral hemisphere whereas paired animals had similar expression in both hemispheres ($*p < 0.05$; ANOVA; $n=10$).

IL-4 knockouts have improved outcome following stroke

To assess the role of IL-4 in the protective effects of social interaction in tissue damage following stroke, WT and IL-4^{-/-} adult male mice were either singly or pair housed for 1 week prior to undergoing MCAO surgery. Brain slices were stained with TTC and infarct volume was analyzed using Inquiry® Quantitative Radiography software. The absence of IL-4 had no effect on infarct volume in isolated mice (Figure 6). However, paired IL-4^{-/-} mice had decreased infarct volumes compared to paired WT mice (Figure 6), indicating a benefit to removing IL-4. Paired IL-4^{-/-} mice also had smaller infarct volumes compared to isolated IL-4^{-/-} mice (Figure 6). The data indicates that IL-4 does not play a necessary role in the protective benefits of social interaction and suggests that reducing IL-4 provides an improved outcome.

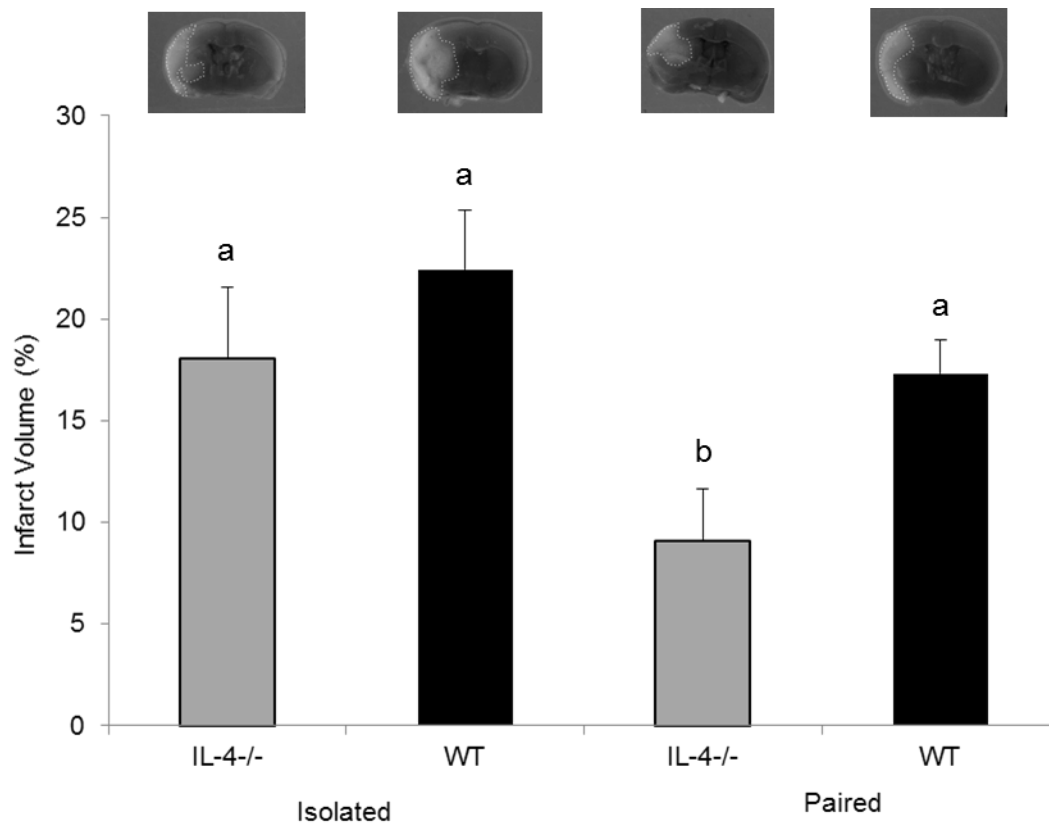


Figure 6. Removing expression of IL-4 results in decreased infarct volume in paired animals. Wild type or IL-4^{-/-} adult male mice were either pair housed or singly housed for 1 week prior to MCAO surgery. Infarct volume was analyzed by TTC staining. Bars that do not share a letter are significantly different from one another. Paired IL-4^{-/-} animals had significantly smaller infarcts than paired WT animals ($p < 0.05$; ANOVA; $n=10$). Paired IL-4^{-/-} also had significantly smaller infarcts than isolated IL-4^{-/-} animals ($p < 0.05$; ANOVA; $n=10$).

CHAPTER 4

DISCUSSION

Investigations into the physiological mechanisms behind the benefits of social interaction have implicated a decrease in inflammation and microglial activation. One potential mediator is oxytocin, which is intimately tied with mammalian social behaviors and has already been shown to reduce infarct volume in a stroke model (Karelina et al., 2011). Because of its established role in reducing microglial activation and as a potent anti-inflammatory cytokine, IL-4 is also a good candidate (Chao et al., 1993). Here, we demonstrate a possible role for IL-4 in the pathways that modulate the physical health benefits of social interaction.

Mice were either pair-housed or singly-housed for one week before analysis of gene expression in the hippocampus, cortex, and hypothalamus. In both the hippocampus and hypothalamus, IL-4 expression was shown to be elevated significantly in paired mice indicating an overall increase in IL-4 production. Following MCAO surgery, paired mice again had higher expression of IL-4 compared to isolated mice. This suggests that social interaction increases production of anti-inflammatory cytokines, even following injury, which could explain the lower risk of illness. Because oxytocin is also known to decrease inflammation, the result provides a potential role for IL-4 in an oxytocin mediated mechanism of neuroprotection.

Previous studies have shown the ability of IL-4 to decrease activated microglia (Chao et al., 1993; Suzumura et al., 1994; Lyons et al., 2009). The overall reduction in activated microglia may decrease peripheral tissue damage following injury to the CNS. This is likely moderated by the CD200-CD200R interaction between neurons and

microglia as a loss of CD200 on neurons results in an increase in activated microglia (Lyons et al., 2007). To examine the effect of IL-4 on neurons, CATH.a cells were incubated with IL-4, and gene expression of CD200 and Crry was analyzed. Following IL-4 administration, expression of CD200 and Crry was increased compared to the vehicle group. This is consistent with previous findings from other research groups showing that intracerebroventricular injection of IL-4 increased CD200 staining in the CNS (Lyons et al., 2007). Our data suggests that the protective effects of social interaction may be induced through this upregulation in CD200 and Crry which mediate microglial activation.

To further examine the interaction between oxytocin and IL-4 with neurons, CATH.a cells were incubated with IL-4 and gene expression of oxytocin, oxytocin receptor (OTR), and IL-4R α was analyzed. IL-4 administration resulted in a non-significant trend towards significance for OTR indicating a possible pathway for IL-4 to control downstream effects of oxytocin. In addition, exogenous IL-4 did not result in a change in oxytocin expressions suggesting that IL-4 may regulate oxytocin signaling through its receptor rather than the actual hormone. IL-4 also downregulated expression of IL-4R α , which may work as a mechanism to maintain equilibrium within the cell. To investigate the effect of oxytocin on neurons, CATH.a cells were incubated with oxytocin and gene expression of IL-4 and IL-4R α was analyzed. Oxytocin had no significant effect on IL-4 expression, although it upregulated IL-4R α expression. Similar to the IL-4 mechanism, oxytocin may regulate downstream effects of IL-4 by regulating expression of its receptor. Interestingly, when oxytocin antagonist was added to block the effect of oxytocin on the cells, IL-4R α expression increased significantly compared to both the

vehicle group and the oxytocin only group. A possible explanation for this unusual effect relies on the fact that oxytocin antagonist is a competitive inhibitor of the oxytocin receptor. Oxytocin likely interacted with the vasopressin receptor to induce IL-4R α expression. Oxytocin and vasopressin have very similar amino acid sequences and structures and have been known to cross-react.

In order to investigate the effect of IL-4 on microglia following stroke, we paired and isolated two groups of mice before performing MCAO surgery on the left hemisphere. Twenty-four hours after the ischemia, microglia were isolated and gene expression of IL-4R α was analyzed. As a comparison, there was no difference in IL-4R α expression on microglia prior to stroke. Post-stroke, within the contralateral hemisphere, paired animals had decreased expression of IL-4R α compared to isolated animals, which could indicate an increase in overall IL-4. This is consistent with the idea that social interaction can decrease pro-inflammatory markers in favor of anti-inflammatory markers. Of the isolated animals, microglia from the contralateral hemisphere expressed higher levels of IL-4R α compared to the ipsilateral hemisphere. This imbalance is not seen in the paired animals; a possible explanation is that following an ischemic event, IL-4 is quickly produced in order to mediate the damage occurring from rampant pro-inflammatory cytokine secretion. Isolated mice which have less circulating IL-4 must produce more IL-4 during these situations.

To determine the importance of IL-4 in the protective effects of social interaction, IL-4^{-/-} mice were housed either with an ovariectomized female or alone for one week. Twenty-four hours following MCAO surgery, tissue was collected and stained with 2,3,5-triphenyltetrazolium chloride. Unexpectedly, knocking out the IL-4 gene seemed to have

a protective effect on infarct volume as pair-housed IL-4^{-/-} mice had significantly smaller infarct volumes compared to paired wild-type mice; our lab and others have demonstrated a similar neuroprotective effect of housing in wildtype mice (Karelina et al., 2011; Karelina et al., 2009a; Karelina et al., 2009b; Venna et al., 2014; O’Keefe et al., 2014; Venna et al., 2012) which suggests that the effects of social housing are not mediated via IL-4. A possible explanation for the reduction in infarct volume caused by knocking out IL-4 is that removal of IL-4 resulted in the upregulation of an alternative pathway. IL-4 has many functions within the immune system which also overlap with the functions of other anti-inflammatory cytokines. It is likely that without IL-4, several other anti-inflammatory cytokines had compensatory effects. Strain differences may also help interpret our results. The C57/Bl6 mice used in our previous experiments have a Th1 phenotypic immune system which has a pro-inflammatory predilection with a focus on pro-inflammatory cytokines such as IL1 β and INF γ . Previous studies demonstrating the protective effects of IL-4 in stroke used BALB/c mice, which have a Th2 phenotypic immune system (Xiong et al., 2011). BALB/c mice produce greater amounts of IL-4 and have an inclination towards anti-inflammatory activity. Because of the reliance on pro-inflammatory cytokines and lower expression levels of IL-4, it is possible that the immune system response of the C57/Bl6 mice to stroke was different than in BALB/c mice and compensated better for the lack of IL-4. In these Th1 mice, IL-4 plays a less important role in the social protection from ischemia-induced damage than in the Th2 mice.

Previous research has indicated the importance of IL-4 in modulating the activation status of microglia. Additionally, because of its potent anti-inflammatory

effects, we suspect that it may play an important role in mediating the neuroprotective benefits of social interaction. In this study, we demonstrate that IL-4 upregulates important neuroprotective markers and can regulate oxytocin expression through the oxytocin receptor. We also show changes in microglial expression of IL-4R α pre- and post-stroke. However, the implications of the study are nebulous considering the seemingly adverse effects of IL-4 in stroke which is contrary to previous literature and seem opposite of what would be suggested by our *in vitro* results. Further elucidating the connection between oxytocin and IL-4 may provide better insight into the mechanism behind the protective effects of social interaction which could lead to improved treatments for stroke in the future.

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